Interleukin-21 activates human natural killer cells and modulates their surface receptor expression

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Summary

Interleukin (IL)-21 is a novel cytokine that has been shown to enhance proliferation and activation of $CD8^+$ T cells, enhance natural killer (NK) cell activity and costimulate anti-CD40-driven B-cell proliferation in mice. Several studies have furthermore demonstrated antitumour effects of IL-21 administration in mouse models. In this study we have investigated how IL-21 affects the survival and cytotoxicity of human NK cells and modulates their expression of surface receptors and of the effector molecules granzyme B and perforin. In contrast to murine NK cells, where IL-21 alone cannot sustain survival, IL-21 and IL-2 were equally efficient in sustaining survival of human NK cells. In the absence of other cytokines, IL-21 had little effect on expression of a panel of surface receptors on human NK cells. However, IL-21 synergized with IL-2 to up-regulate several surface receptors, including NKG2A, CD25, CD86 and CD69. The $CD25^+$ $CD86^+$ NK cells were $CD56^{bright}$ and were large and granular. Expression of the effector molecules perforin and granzyme A and B was up-regulated by IL-21 at both mRNA and protein levels. Furthermore, IL-21 increased the cytotoxicity of NK cells against K562 target cells. These findings suggest that IL-21 modulates NK cell activity through induction of intracellular effector molecules as well as modulation of surface receptor expression.

Keywords: cytokines; natural killer cells; cell activation; cell surface molecules

Introduction

Interleukin (IL)-21 is a recently discovered cytokine secreted by activated $CD4^+$ T cells and natural killer T (NKT) cells.[1,2] Its sequence, protein structure and gene structure place it in the common γ-chain-dependent family of cytokines, with greatest similarity to IL-2 and IL-15. Like these cytokines, IL-21 recruits the common γ-chain which forms heterodimers with the IL-21-specific receptor protein (IL-21R).[1]

IL-21R is expressed by most mononuclear cells and is up-regulated on NK, B and T cells after activation.[1,3] This pattern of expression suggests that the primary effects of IL-21 are immunomodulatory, a hypothesis that has been confirmed by *in vitro* and *in vivo* analyses. The activities of IL-21 include effects on both lymphoid and myeloid cell lineages. IL-21 stimulation of T, B and NK cells leads to enhanced proliferation and mature effector function.[4]

IL-21 has been reported to increase IL-2- or IL-15-induced murine NK cell activation, cytotoxicity and production of IFN-γ and IL-10,[5] and IL-21 was also found to increase the cytotoxicity of *in vivo* preactivated splenic NK cells, but did not enhance the cytotoxicity of naïve NK cells.[6] However, IL-21 alone was not able to keep purified murine bone marrow NK cells alive, and IL-21 decreased IL-15-mediated expansion of NK cells.[6] In humans, IL-21 is an important differentiation factor for NK cells. Accordingly, the addition of IL-21 and IL-15 to $CD34^+$ haematopoietic stem cells from bone marrow or cord blood supplemented with stem cell factor (SCF) and FMS-like tyrosine kinase 3 ligand (Flt3L) promoted the generation of NK cells, leading to the generation of mature NK cells highly capable of killing major histocompatibility complex (MHC)-deficient target cells.[1,7] By contrast, IL-21 does not appear to be important for the development of murine NK cells, as these are normal in

number and function in IL-21R-deficient mice.[6] From these studies it is clear that IL-21 has different effects on murine and human NK cells.

Several publications have shown an enhanced antitumour response in mouse models upon IL-21 treatment.[5,8–12] $CD8^+$ T cell- and NK cell-dependent mechanisms both contribute to the antitumour activity of IL-21 depending on the model system. Thus, Moroz *et al*. showed that IL-21 treatment strongly increased memory $CD8^+$ T-cell responses and prolonged survival of mice injected with E.G7 lymphoma cells.[11] In another study using the B16-F10 and RenCa tumour models, hydrodynamic injection of an IL-21 plasmid strongly reduced the number of lung metastases in an NK cell- and perforin-dependent manner, as shown by depletion studies and by the use of gene-targeted mice.[5]

Although the effects of IL-21 on murine NK cells have been extensively studied *in vitro* as well as *in vivo*, little is known about the IL-21-mediated effects on human NK cells. IL-21 has recently entered clinical trials in stage IV metastatic melanoma and was demonstrated to be safe and well tolerated.[13] Hence, it is important to understand how IL-21 acts on the human immune system in order to fully exploit the potential of IL-21 for human therapy. Here, we have investigated how IL-21 affects the survival, activation and surface receptor expression of primary human NK cells.

Materials and methods

Cell culture

Blood was drawn from healthy volunteers, after informed consent had been obtained, into heparin- or citrate-coated CPT tubes (BD Biosciences, San Jose, CA) and peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation according to the manufacturer's recommendation. PBMC were washed twice with phosphate-buffered saline (PBS) and cultured in RPMI-1640 with GlutaMAX (Invitrogen, Carlsbad, CA), 10% heat-inactivated fetal calf serum (FCS), 50 μM 2-mercaptoethanol and antibiotics. Human recombinant IL-2 (Peprotech, Rocky Hill, NJ or BD Biosciences) and IL-21 (Zymogenetics, Seattle, WA) were added to the cultures as indicated in the figure legends. For cells cultured for more than 3 days, medium was always replaced with fresh medium and cytokines on day 3 and, in some experiments, also on day 6. All experiments using human material were approved by the Danish Ethical Committee.

Purification of NK cells

PBMC were purified as described above, and pure NK cells were obtained by one of two protocols: (1) depletion of $CD3^+$ cells with directly conjugated microbeads followed by positive purification of $CD56^+$ cells with microbeads or (2) negative purification with NK Cell Isolation Kit II (all beads were from Miltenyi Biotech, Bergisch Gladbach, Germany). Purification was performed manually or with an AutoMACS (Miltenyi). The purity of the cells was always controlled by fluorescence-activated cell sorting (FACS) and was more than 90%. For the isolation of CD56 dim and bright subsets, the magnetic antibody cell sorting (MACS)-purified $CD3^-$ $CD56^+$ NK cells were labelled with CD56 antibody and sorted into $CD56^{bright}$ and $CD56^{dim}$ populations on a FACSDiva (BD Biosciences). Purified cells were cultured under the same conditions as described above for PBMC.

Reagents and flow cytometry

The following antibodies and reagents were purchased from BD Biosciences: CCR7 phycoerythrin (PE)-Cy7 (clone 3D12), CD3 PerCP (clone SP34-2), CD4 allophycocyanin (APC)-Cy7 (clone RPA-T4), CD8 fluorescein isothiocyanate (FITC) or PE-Cy7 (clone RPA-T8), CD16 APC-Cy7 or FITC (clone 3G8), CD40 FITC (clone 5C3), CD45 FITC (clone HI30), CD56 PE-Cy7 (clone B159), CD86 PE (clone IT2·2), CD94 APC (clone HP-3D9), CD154 PE (clone TRAP1), CD158a FITC (clone HP-3E4), CD158b PE (clone CH-L), human leucocyte antigen (HLA)-DR FITC (clone G46-6), NKG2D APC (clone ID11), CD161 APC (clone DX12), NKp44 PE (clone Z231), NKp46 PE (clone 9E2/NKP46), perforin PE (clone dG9), granzyme B FITC (clone GB11), annexin V, propidium iodide and brefeldin A. CD25 FITC (clone B1·49·9), CD56 APC (clone N901) and NKG2A PE (clone Z199) were purchased from Beckman Coulter (Fullerton, CA). Cells were stained for 30 min with the appropriate antibodies, washed twice and analysed on a FACSCanto (BD Biosciences) using the BD FACSDIVA software, version 4·1.2. For intracellular staining of perforin and granzyme (grz) B, brefeldin A (2 μg/ml) was added to the cultures 4 hr before harvest. Intracellular staining was performed using the Cytoperm/Cytofix kit (BD Biosciences) according to the manufacturer's recommendation. Measurements of apoptotic and necrotic cells were performed after 3 and 7 days of culture using annexin V and propidium iodide staining followed by flow cytometry, and the percentage of live cells was calculated as the percentage of cells that remained unstained. In most experiments, acquisition was continued until 5000 $CD56^+$ $CD3^-$ NK cells had been collected for analysis.

Quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted from purified cells using the Mini RNA Isolation Kit from ZymoResearch (Orange, CA). cDNA was prepared from each of the purifications using random primers and TaqMan reverse transcription

reagents (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Quantitative PCR was performed in duplicate on each of the cDNA samples (10-fold dilutions of cDNA) using TaqMan PCR core reagents (Applied Biosystems) and the ABI Prism® 7900HT Sequence Detection System (Applied Biosystems). Primers and 6-carboxyflourescein (FAM)-labelled probes for grz A mRNA, grz B mRNA, perforin mRNA, and 18S rRNA were ordered as Assays-on-Demand (Applied Biosystems). Probe sequences for these assays were as follows: grz A, TCCTGCTAATTCCTGAAGATGT CTG; assay Hs00196206_m1; grz B, CTTCCTTTAAGGG GGACTCTGGAGG; assay Hs00188051_m1; perforin, GAGTGCCGCTTCTACAGTTTCCATG; assay Hs001 69473_m1; and 18S rRNA, TGGAGGGCAAGTCTGGT GCCAGCAG; assay HS99999901_s1. Data were analysed using ABI PRISM SDS 2·2 software (Applied Biosystems), and expression levels for grz A, grz B, and perforin-1 were normalized to the 18S rRNA levels.

Cytotoxicity assays

Target cells. K562 cells were cultured in RPMI-1640 with the addition of 10% FCS, and split 1 : 2 the day before the assay to ensure exponential growth. Two to 10 million K562 cells were pelleted by centrifugation and re-suspended in 100 µl of $Na^{51}CrO_4$ at 5 µCi/µl (Amersham, Buckinghamshire, UK) in PBS and incubated for 1 hr at 37°. The cells were washed twice in medium and used within 1 hr as target cells in the cytotoxicity assay. Daudi cells were cultured and labelled as for K562 cells except that twice the amount of ^{51}Cr was used to label the cells.

Effector cells. Human PBMC were isolated and cultured as described above. The cells were washed twice and counted immediately before use as effector cells.

Cytotoxicity assay. Target and effector cells were plated in round-bottom 96-well plates at 5000 target cells per well and incubated for 4 hr. Effector:target ratios were as stated in the figure legends. The assay was always run in triplicate, and measurements of spontaneous release (target cells only) and maximum release (target cells lysed with 5% Triton X-100) were repeated six times. The cells were briefly pelleted and 100 µl of supernatant was removed for measurement of ^{51}Cr release in a Packard Cobra gamma counter (GMI, Ramsey, MN). Specific lysis was calculated as [(sample lysis – spontaneous lysis)/(maximum lysis – spontaneous lysis)] × 100%.

Statistical analysis

All statistical analyses were performed using a paired Student's *t*-test.

Results

IL-21 promotes survival of human NK cells

Previous studies suggest that IL-21 may have different effects on murine and human NK cells.[1,5,14] To our knowledge, it has not been investigated whether IL-21 can support survival of human NK cells. To address this question, purified human NK cells were cultured in medium alone or with IL-21, IL-2 or both cytokines in combination. Apoptotic and necrotic cells were measured after 3 and 7 days of culture using annexin V and propidium iodide staining. We found that IL-21 promoted the survival of the NK cells to the same extent as IL-2, and there was no synergistic effect of having both cytokines in the culture (Fig. 1a and b). Furthermore, the three doses of IL-21 that were tested (10, 25 and 100 ng/ml) had similar effects. As only around 30% of the cells were alive after 7 days of culture in cytokines, and less than 10% were alive when cells were cultured without cytokines, most of the subsequent experiments were performed with whole PBMC cultures, where survival is much higher (data not shown).

IL-21 activates NK cells

After 3 days of culture, expression of the early activation marker CD69 on NK cells was up-regulated by both IL-2 [mean ± standard error of the mean (SEM) 17·2 ± 6·1%] and IL-21 (12·3 ± 1·9%) compared with cultures without added cytokines (5·2 ± 0·8%). A strong up-regulation of CD69 (45·9 ± 9·1%) was observed when both cytokines were added to the cultures, suggesting a synergistic effect on activation of NK cells (Fig. 2a). In contrast, HLA class II expression was induced by IL-2 but not by IL-21. Another activation marker, CD25 (IL-2Rα), was barely detectable in any of the cultures at day 3 (data not shown). After 7 days in culture with both IL-2 and IL-21, CD69 expression was further increased (data not shown)

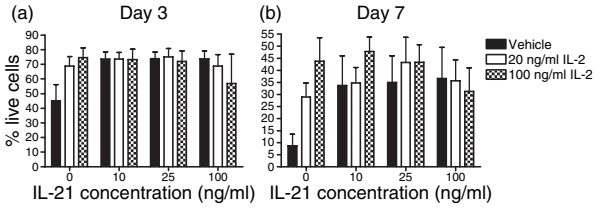

Figure 1. Interleukin (IL)-21 promotes survival of natural killer (NK) cells *in vitro*. NK cells were purified by magnetic antibody cell sorting (MACS) purification of $CD3^-$ $CD56^+$ cells and cultured for 3 days (a) or 7 days (b) with IL-21 and/or IL-2 as indicated. Apoptosis and necrosis were measured using annexin V and propidium iodide staining. The graph represents data from five or six donors obtained in five separate experiments. $^*P < 0{\cdot}05$ versus unstimulated cells.

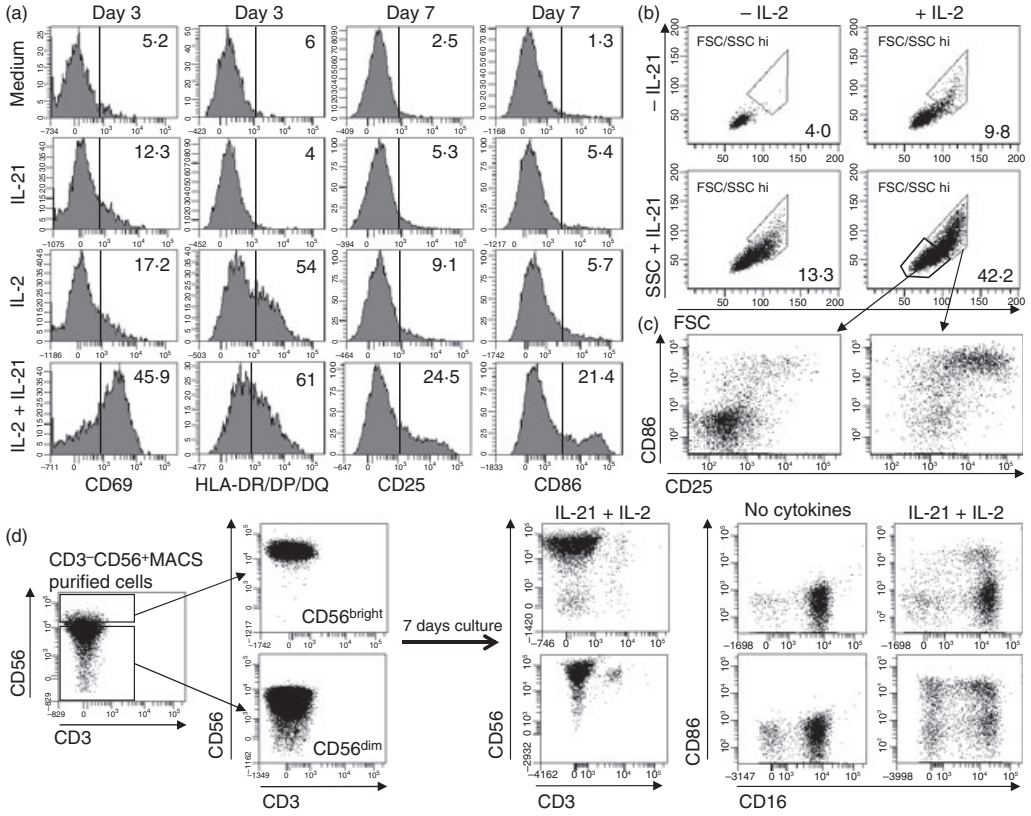

Figure 2. Interleukin (IL)-21 induces activation and maturation of natural killer NK cells. Freshly isolated human peripheral blood mononuclear cells (PBMC) were cultured for 3 or 7 days with IL-21 (10 ng/ml) or IL-2 (20 ng/ml) as indicated and the expression of activation markers was analysed by flow cytometry. All charts were gated on $CD3^-$ $CD56^+$ NK cells. The charts show data from one representative donor of three to 11 donors performed in two to four experiments. The numbers denote the percentage of positive cells as an average for all donors. (a) Expression of CD69 (day 3) and CD25 and CD86 (day 7) was increased by IL-21, whereas human leucocyte antigen (HLA) class II (day 3) was increased by IL-2 only. Expression of CD69 and HLA class II was further increased on day 7 (data not shown). (b) Forward and side scatter plots of gated NK cells. Both FSC and SSC increased upon IL-21 stimulation. (c) The front scatter (FSC)/side scatter (SSC)^{low} cells are mainly $CD25^-$ $CD86^-$, whereas the FSC/SSC^{high} cells are mainly $CD25^+$ $CD86^+$. (d) Magnetic antibody cell sorting (MACS) purified $CD3^-$ $CD56^+$ NK cells were sorted into $CD56^{bright}$ and $CD56^{dim}$ populations and cultured for 7 days with cytokines as above. After 7 days in culture with IL-2 + IL-21, the $CD56^{dim}$ cells up-regulated CD56 expression, and both subsets expressed CD86 on a fraction of both $CD16^+$ and $CD16^-$ cells. The data are for one representative donor of five.

and CD25 was strongly up-regulated (24·5 ± 7·5%), whereas either cytokine alone induced only low CD25 expression on the NK cells (IL-21: 5·3 ± 1·8%; IL-2: 9·1 ± 2·1%) and almost no CD25 was detected on NK cells cultured without cytokines (2·5 ± 0·9%) (Fig. 2a).

The costimulatory ligand CD86 (B7-2), which is normally not expressed by NK cells, was also induced by the combination of IL-2 and IL-21 with a similar pattern to that for CD25 (Fig. 2a). Co-staining showed that CD25 and CD86 were up-regulated on the same cells. Analysis of the front scatter (FSC)/side scatter (SSC) properties of the NK cells revealed that a fraction of the NK cells became large and highly granular after 7 days in culture with both cytokines, and this fraction also contained the $CD25^+$ $CD86^+$ cells (Fig. 2b). An increase in size and granularity is normally a hallmark of lymphocyte activation, suggesting that IL-21 and IL-2 synergistically activated a fraction of the human NK cells.

After 7 days of culture of PBMC with both cytokines, almost all NK cells were $CD56^{bright}$. Therefore, it was not clear whether CD25 and CD86 were induced on the $CD56^{bright}$ or $CD56^{dim}$ subset or on both subsets. We speculated that the high CD56 expression could have been a result either of a preferential expansion of the $CD56^{bright}$ subset or of up-regulation of CD56 on the $CD56^{dim}$ subset. To examine these hypotheses, we isolated NK cells by MACS purification and sorted them into $CD56^{bright}$ and $CD56^{dim}$ subsets which were then cultured for 7 days with cytokines as above. We found that the $CD56^{dim}$ subset up-regulated CD56 after IL-2 culture. CD86 was expressed by both subsets and by both $CD16^+$ and $CD16^-$ cells, suggesting that the ability to up-regulate CD86 is not limited to either the $CD56^{bright}$ or the $CD56^{dim}$ subset (Fig. 2c). As both subsets greatly increased FSC and SSC upon stimulation (data not shown), the results suggest that IL-21 and IL-2 synergistically can activate both subsets.

IL-21-mediated effects on the expression of NK cell receptors

In mice, IL-21 has been shown to modulate the expression of several activating and inhibiting NK cell receptors on purified IL-2-cultured NK cells. IL-21 increased the expression of CD94, NKG2A/C/E and KLRG1 but decreased the expression of Ly49D, Ly49F and NK1·1, whereas expression of other receptors was unaffected by IL-21.[5] In the present study, we examined whether IL-21 also modulated the expression of surface markers in human NK cells in PBMC cultures.

IL-21 alone, but not IL-2, was able to induce a moderate increase in the surface level of the natural cytotoxicity receptor (NCR) NKp46, which is constitutively expressed by NK cells (Fig. 3 and Table 1), whereas expression of another NCR, NKp44, which is not expressed on resting NK cells, was not changed by IL-21. Compared with cultures without cytokines or with IL-2 alone, addition of IL-21 slightly but consistently increased the fraction of NK cells expressing the inhibitory receptors CD94 and NKG2A. The surface level of the activating receptor NKG2D was also slightly up-regulated by IL-21, although IL-2 induced a larger increase in NKG2D, and there was no additive effect of IL-21 and IL-2 (Fig. 3). Similar results were obtained after 7 days of culture (data not shown). The receptor 2B4 (CD244) was slightly up-regulated by both IL-21 and IL-2. This receptor has both inhibitory and activating functions and has been shown to enhance IL-2-mediated NK cell proliferation.[15,16] Finally, we did not find any changes in the surface level of CD158a (KIRDL1) (Fig. 3), CD158b (KIRDL2/3), CD161 (NKR-p1c), CD62L (L-selectin) or CXCR3 induced by IL-21 after 3 or 7 days of culture (data not shown). Thus, IL-21 had only moderate effects on the surface level of inhibitory and activating NK cell receptors in human NK cells and, in contrast to murine NK cells, IL-21 did not decrease the expression of CD161 (NK1·1).[5]

IL-21 increases expression of effector molecules

A previous report by Brady *et al.* showed increased expression of perforin in NK cells from $Rag^{-/-}$ mice cultured with IL-21 and IL-15.[5] We wanted to investigate whether IL-21 was able to augment perforin and grz A and B mRNA expression in human NK cells. Purified NK cells were cultured for 72 hr with IL-2 and IL-21 alone or in combination, and the levels of perforin, grz A and grz B mRNA were measured by quantitative reverse transcription–polymerase chain reaction (RT-PCR). Expression of both perforin and grz A and B was consistently up-regulated by both cytokines (1·5–4-fold up-regulation), and the highest expression levels of perforin and grz B were reached when both cytokines were added, whereas there was no additive effect on grz A (Figs 4a–c).

We next analysed the intracellular protein levels of perforin and grz B by flow cytometry on PBMC stimulated with cytokines for 1–3 days. IL-21 stimulation consistently resulted in a small, but statistically significant, increase in the mean fluorescence intensity of grz B ($P < 0{\cdot}05$ for IL-21 versus none; $P < 0{\cdot}01$ for IL-21 + IL-2 versus IL-2) and perforin ($P < 0{\cdot}01$ for IL-21 versus none; $P < 0{\cdot}001$ for IL-21 + IL-2 versus IL-2), whereas IL-2 did not have any effect (Figs 4d–e). Thus, IL-21 was able to increase both mRNA and protein expression of the effector molecules perforin and grz B.

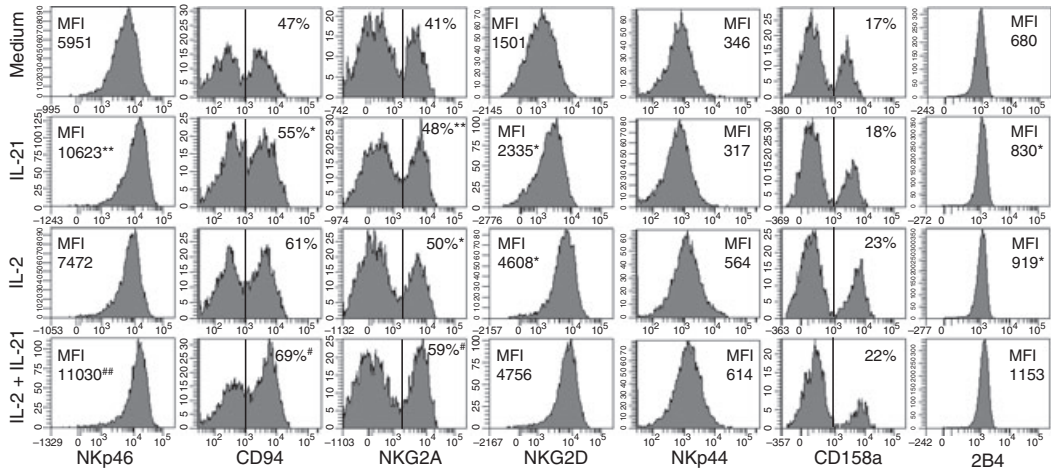

Figure 3. Surface phenotype of natural killer (NK) cells upon interleukin (IL)-21 stimulation. Peripheral blood mononuclear cells (PBMC) were stimulated as described in Fig. 2 and analysed for their surface marker phenotype on day 3. All charts were gated on $CD3^-$ $CD56^+$ NK cells. The charts show data from one representative donor of five to eight donors performed in two to three experiments. The numbers denote either the average mean fluorescence intensity of all donors (NKp46, NKG2D, NKp44 and 2B4) or the average percentage of positive cells (CD94, NKG2A and CD158a). $^*P < 0{\cdot}05$ and $^{**}P < 0{\cdot}01$ versus unstimulated cells; $^{\#}P < 0{\cdot}05$ and $^{\#\#}P < 0{\cdot}01$ versus IL-2 only.

Table 1. Expression of natural killer (NK) cell receptors after interleukin (IL)-2 and IL-21 stimulation

IL-2 (ng/ml)	IL-21 (ng/ml)	% positive cells on day 3 [mean (range)]								
		CD158a ($n = 5$)	CD158b ($n = 2$)	CD62L ($n = 6$)	CXCR3 ($n = 6$)	CD94 ($n = 5$)	NKG2A ($n = 8$)	CD69 ($n = 6$)	HLA-DR ($n = 3$)
0	0	16·6 (8·9–31·3)	31·1 (22·4–39·7)	35·3 (22·5–40·8)	34·1 (16·7–41·3)	46·5 (37·8–53·8)	40·6 (30·5–55·1)	5·2 (2·7–7·8)	5·6 (3·1–10·5)
20	0	18·2 (11·7–27·7)	28·1 (20·7–35·4)	36·8 (28–44·3)	40·6 (19·7–54·4)	55 (49·6–63·8)	48·1 (37·7–64·2)	12·3 (7·4–19·5)	4 (2·9–5·5)
0	10	22·8 (12·4–31·2)	26·7 (20·2–33·1)	30·5 (16–38·1)	21·9 (10·1–29·4)	60·8 (44·8–87·7)	50·4 (32·3–74·5)	17·2 (4·2–46·5)	54·3 (39·1–76·4)
20	10	22 (8·4–33·4)	19·1 (12·7–25·4)	32 (22·7–35·8)	18·9 (8·8–29·8)	68·6 (56·7–95·3)	59·1 (39–83·3)	45·9 (12·2–72·8)	60·7 (45·4–79·9)

IL-2 (ng/ml)	IL-21 (ng/ml)	% positive cells on day 7 [mean (range)]		MFI (range) on day 3					
		CD25 ($n = 8$)	CD86 ($n = 11$)	NKG2D ($n = 8$)	NKp44 ($n = 3$)	NKp46 ($n = 5$)	CD161 ($n = 5$)	CD244 ($n = 6$)	CD158a ($n = 5$)
0	0	3·4 (0·5–8·5)	1·4 (0·2–3·2)	1501 (662–3567)	346 (273–433)	5951 (4435–7220)	1163 (407–2341)	680 (437–842)	16·6 (8·9–31·3)
20	0	6·2 (1·6–13·6)	4·5 (0·9–12·8)	2335 (914–6083)	317 (237–375)	10623 (8290–14289)	1470 (313–3732)	830 (481–1078)	18·2 (11·7–27·7)
0	10	10·4 (2·8–19·7)	5·4 (1·2–12)	4608 (1801–14916)	564 (409–836)	7472 (4934–9613)	1951 (409–3985)	919 (462–1299)	22·8 (12·4–31·2)
20	10	24·5 (5·7–51·6)	17 (4–45·7)	4756 (1944–14081)	614 (378–958)	11030 (6789–15668)	1306 (212–2751)	1153 (593–2019)	22 (8·4–33·4)

HLA, human leucocyte antigen; MFI, mean fluorescence intensity.

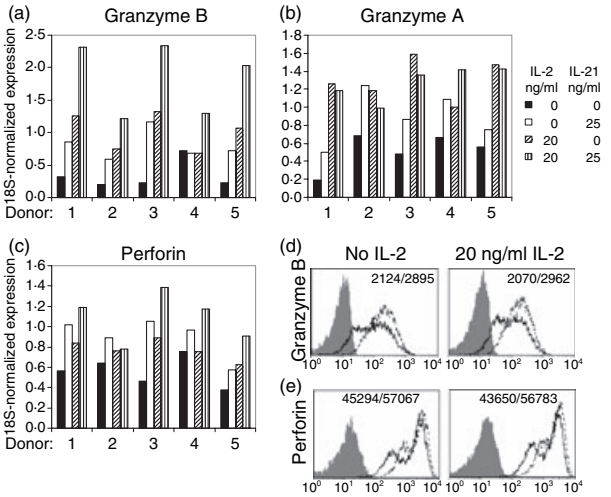

Figure 4. Interleukin (IL)-21 induces perforin and granzyme (grz) and B expression. Purified natural killer (NK) cells were cultured for 72 hr with the indicated combinations of IL-21 (25 ng/ml) and IL-2 (20 ng/ml) and subsequently analysed for grz A and B and perforin expression. (a–c) $CD3^-$ $CD56^+$ NK cells were isolated from the cultures and analysed for grz B (a), grz A (b) and perforin (c) mRNA expression by quantitative reverse transcription–polymerase chain reaction (RT-PCR). The data are for five donors and values have been normalized to 18S expression. (d–e) Peripheral blood mononuclear cells (PBMC) were cultured as above and analysed by flow cytometry for intracellular grz B (d) and perforin (e) protein levels (no IL-21, solid line; 25 ng/ml IL-21, dashed line; isotype, filled region). Numbers denote average mean fluorescence intensity with/without IL-21, respectively, for eight donors and three independent experiments. Similar results for protein expression were obtained after 2 and 3 days of stimulation (data not shown).

IL-21 increases NK cell cytotoxicity *in vitro*

The finding that IL-21 increased the expression of activation markers as well as effector molecules in NK cells suggests that IL-21 may increase the cytotoxicity of NK cells. Previous data from both mice and humans have shown that IL-21 is indeed able to augment *in vitro* cytotoxicity of NK cells.[17] In the light of these results, we wanted to carefully analyse the effect of IL-21 dose, time of stimulation of the NK cells, and combination with IL-2 on NK cell-mediated cytotoxicity in human PBMC cultures. The widely used MHC-deficient cell line K562 was chosen as the target cell. The results showed that (1) 10, 25 and 100 ng/ml of IL-21 were equally effective; (2) 24, 48 and 72 hr of stimulation of the PBMC resulted in similar cytotoxicity, and (3) there was no synergistic effect of IL-21 and high-dose IL-2 (100 ng/ml) (Figs 5a and c). However, at lower IL-2 concentrations, IL-21 still increased the cytotoxicity, suggesting an additive effect of IL-21 and suboptimal IL-2 (Fig. 5b). Similar results were obtained when purified NK cells were used as effector cells (data not shown).

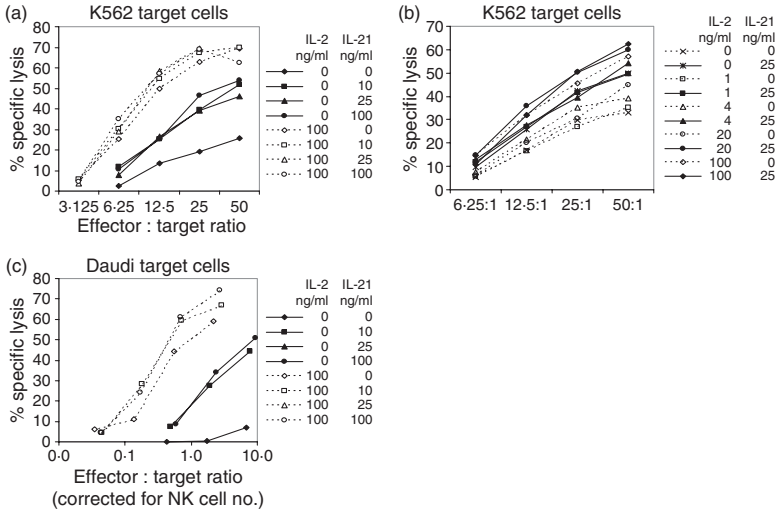

Figure 5. Interleukin (IL)-21 augments natural killer (NK) cell cytotoxicity. Peripheral blood mononuclear cells (PBMC) were cultured with the indicated amounts of IL-2 and IL-21 and subsequently used as effector cells in a ^{51}Cr-release assay. (a, b) Cytotoxicity against K562 target cells after 18 hr of stimulation at varying IL-21 concentrations (a) and at varying IL-2 concentrations (b). Data are averages for four donors. (c) Cytotoxicity against Daudi target cells after 7 days of stimulation. Data are for three donors.

We next investigated whether a longer stimulation with IL-21 could modify NK cell killing. PBMC were cultured for 7 days with IL-21 and IL-2 alone or in combination prior to use of the cells as effectors against Daudi cells, which are resistant to natural NK cytotoxicity but sensitive to IL-2-cultured NK cells. As with the K562 cells, all doses of IL-21 were equally effective, and there was no synergy with IL-2 (Fig. 5c).

Discussion

Since the discovery of IL-21 was first reported in 2000,[1] several studies have highlighted the pleiotropic nature of IL-21 and demonstrated NK cell-dependent and cytotoxic T cell-dependent antitumour effects of IL-21. IL-21 has been shown to modulate expression of surface receptors expressed by NK cells, and to regulate cytokine production and cytotoxicity of purified, IL-2-cultured murine NK cells.[5] In this study we have investigated the effects of IL-21 on human NK cells alone and together with IL-2. Both cytokines were able to sustain the survival of purified NK cells compared with cultures without added cytokines, although survival was low after 7 days of culture. Coquet *et al.* recently reported a similar effect of IL-21 on survival of NKT cells, although IL-2 was superior to IL-21 in promoting survival of NKT cells.[2] We found that IL-21 was not as powerful as IL-2 as an inducer of NK cytotoxicity. However, IL-21 had different effects on the expression of NK surface receptors compared with IL-2. NKp46, an NCR, was up-regulated only by IL-21, whereas the two activating receptors NKG2D and NKp44 were up-regulated mainly or exclusively by IL-2. NKp46 has been described as a major triggering receptor involved in killing of autologous targets, and a correlation between NKp46 and the ability to kill target cells has been demonstrated.[18] It is thus possible that up-regulation of NKp46 might lead to the IL-21-mediated increase in cytotoxicity, whereas IL-2 seems to use a different pathway as IL-2 had no effect on NKp46 expression.

The killer-cell immunoglobulin-like receptor (KIR) family is involved in regulation of NK cell activity.[19] We found that IL-21 did not change the fraction of NK cells expressing KIR2DL1 (CD158a; Fig. 3a) and KIR2DL2/3 (CD158b; data not shown). These receptors bind to HLA-C alleles, leading to inhibition of NK cell activation. In mice IL-21 decreased the fraction of NK cells expressing Ly49f and d, molecules that are functionally homologous to the human KIR family.[5] These results suggest that IL-21 has different effects on murine and human NK cells.

In a study by Burgess *et al.*, IL-21 was recently reported to down-regulate the expression of the activating receptor NKG2D and one of its downstream targets, DAP10, in primary human NK cells.[14] In contrast to these results, a small IL-21-mediated increase in NKG2D expression was observed in the present study, which was not further augmented after 7 days of culture (data not shown). Although the biological importance of this increase in NKG2D expression may be minor it is clear that there was no down-regulation of NKG2D. It is possible that factors produced by accessory cells in the PBMC cultures could have inhibited or reversed IL-21-mediated down-regulation of NKG2D in our studies, as opposed to the study by Burgess *et al.*, where purified NK cells were used. To our knowledge, the ability of IL-21 to modulate NKG2D expression in murine NK cells *in vitro* has not been described. One paper showed that, in mice treated with the NKT cell-activating agent α-galactosylceramide, hydrodynamic delivery of IL-21 plasmid 3 days later did not change NKG2D expression of the NK cells.[20] Also, IL-21 did not affect NKG2D expression in IL-2-cultured murine NKT cells.[2] However, it is clear that the NK cell-mediated antitumour effect of IL-21 depends upon interaction between NKG2D on the NK cells and its ligands expressed by the tumour cells[21] which may be somewhat

in contrast to the inability of IL-21 to up-regulate NKG2D.

Additive effects of IL-21 and IL-2 on several NK cell receptors were observed. IL-21 mediated a small but consistent increase in CD94, NKG2A and 2B4 (CD244), in both the presence and the absence of IL-2. As the increase was small it is not clear whether this has any biological significance. The up-regulation of CD94 and NKG2A is consistent with reported studies on murine NK cells and NKT cells,[2,5] although IL-21-induced up-regulation of these markers was much higher on murine NK cells, leading to expression of these receptors in all NK cells. In the same study, IL-21 was also reported to strongly increase perforin protein expression as well as increasing IL-2- and IL-15-induced NK cytotoxicity against different target cells. It appears that IL-21 drives both murine and human NK cells towards maturation and activation, but IL-21 has more pronounced effects on murine NK cells. The mouse studies were performed with purified bone-marrow or splenic NK cells cultured for 7 days, whereas we used human NK cells from the blood; hence, it is possible that differences in the source of NK cells could influence the results. Such a difference was found in a recent paper showing that murine blood- and spleen-derived NK cells differ in their maturation and expression of CD27.[22] The use of purified NK cells versus PBMC cultures might also have influenced the results. In support of this notion, a study by Gays *et al.* showed that IL-21 mediates down-regulation of NKG2 and CD94 on NK cells in IL-2-cultured splenic cells,[23] which is in contrast to the up-regulation of NKG2A and CD94 observed in cultures of purified splenic NK cells from $RAG^{-/-}$ mice.[5] Thus, the presence of accessory cells might modulate the overall effect of IL-21.

After 7 days of culturing with both IL-2 and IL-21, most of the activated $CD56^{+}$ $CD3^{-}$ cells coexpressed CD25 and the costimulatory ligand CD86. Expression of CD86 has previously been described on NK-92 cells as well as on IL-2 + phytohaemagglutinin (PHA)-activated primary human NK cells, whereas naïve NK cells do not express CD86.[24,25] This suggests that the expression of CD86 is associated with an activated phenotype, although the function of CD86 on NK cells is unknown. The finding that NK cells also expressed HLA-DR after IL-2 activation suggests that they may be capable of presenting antigens to and activating $CD4^{+}$ T cells. Recently, a novel cell type termed 'interferon-producing killer dendritic cells (IKDC)' with a molecular expression profile characteristic of both NK cells and dendritic cells was described in mice.[26,27] We speculate that our CD86-expressing NK cells might represent a human counterpart of the murine IKDC; more studies will be needed to investigate this possibility.

The developmental relationship between $CD56^{bright}$ and $CD56^{dim}$ cells is not yet clear. The $CD56^{bright}$ cells predominate in lymph nodes and in cultures after cytokine (IL-12 and IL-15) stimulation, whereas the $CD56^{dim}$ subset predominates in blood and is more cytolytic.[28] Our findings suggest that $CD56^{dim}$ cells can be turned into $CD56^{bright}$ cells, but the fact that these cells are highly cytolytic suggests that they may not necessarily be identical to the naturally occurring $CD56^{bright}$ cells.

Several lines of evidence support the notion that IL-21 can activate human NK cells. First, we observed an increase in size and granularity based on FSC/SSC analysis after 7 days of culture in both IL-21 and IL-2, consistent with previously reported results.[5,14] Secondly, the combination of IL-2 and IL-21 led to up-regulation of the activation marker CD69 after 3 days of culture and up-regulation of CD25 after 7 days of culture. Both of these markers were barely detectable on freshly isolated NK cells. Thirdly, IL-21 increased mRNA expression of the effector molecules perforin, grz A and grz B, as well as protein expression of grz B and perforin, all of which are involved in target cell killing.[29] Thus, IL-21 has previously been shown to enhance the antitumour activity of NK cells in a perforin-dependent manner.[5] Finally, IL-21 increased the cytotoxicity of the NK cells against K562 and Daudi target cells. All these data suggest a role of IL-21 in augmenting the activation of NK cells, which is consistent with the previously published NK cell-dependent antitumour effects of IL-21 observed in mice.[5,12,21,30] In conclusion, our data support the notion that IL-21 can activate human NK cells, suggesting that IL-21 might be applied therapeutically to enhance NK cell-mediated antitumour responses. These results should further encourage the testing of IL-21 as a potential novel oncology drug.

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